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APPLICATION NUMBER: *60/556,000*
FILING DATE: *March 24, 2004*
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

22386 U.S.PTO 60/556000 032404

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
L. D.	McCullough	

☒ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

NOVEL METHOD OF NEUROPROTECTION BY PHARMACOLOGICAL INHIBITION OF AMP-ACTIVATED PROTEIN KINASE

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 13 ☐ CD(s), Number [ ]

☐ Drawing(s) *Number of Sheets* 6 ☒ Other (specify) Claims (1 page)

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

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Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME **N. Whitney Wilson**

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Date **3/23/04**

REGISTRATION NO. *(if appropriate)* **38,661**

Docket Number: **C041103/0125008**

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Number 2 of 2

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Provisonal Application of:)
L.D. McCullough, *et al.*)
Filed:March 23, 2004)
For: **NOVEL METHOD OF**)
NEUROPROTECTION BY)
PHARMACOLOGICAL)
INHIBITION OF AMP-ACTIVATED)
PROTEIN KINASE

CERTIFICATE OF EXPRESS MAILING

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Dewayne Franklin
General Services Department
John Mathews

Novel Method of Neuroprotection by Pharmacological Inhibition of AMP-activated Protein Kinase

Background of the Invention

Stroke, defined as an abnormality in brain function resulting from disruption of cerebral circulation, is one of the leading causes of death in the U.S. Even when a stroke does not result in death, the costs it imposes on the victim, physical, emotional, and financial, can be staggering. These costs stem from the tremendous damage done to the victim's brain by the stroke. With a reduction in oxygen and glucose, cells display a rapid disruption of protein synthesis, depletion of intracellular energy stores, destabilization of the cell membrane, and activation of the NMDA receptor, leading to excitotoxic and oxidative cell damage in the brain. In an attempt to survive and repair the oxidative damage and return the cell to homeostasis, numerous compensatory energy-consuming processes are activated. However, over-activation of these pathways can deleterious, further depleting cellular energy, and resulting in further brain damage. Such brain damage is, generally, irreversible. Accordingly, a method of protecting brain tissue from damage during a stroke (neuroprotection) would be tremendously important.

AMP-activated protein kinase (AMPK), a member of a metabolite-sensing protein kinase family, is a known sensor of peripheral energy balance (Carling D., "The AMP-activated protein kinase cascade- a unifying system for energy control." *Trends Biochem Sci* 6:314 (2): 580-585, 2004.) AMPK is a heterotrimeric protein composed of a catalytic α subunit (α1 or α2), and 2 regulatory subunits (β and γ) (6). AMPK is phosphorylated and activated when cellular energy levels are low. AMPK in turn

regulates cellular metabolism and chronically regulates gene expression to restore ATP levels. Increases in the AMP/ATP ratio, changes in cellular pH and redox status, and increases in the creatine/phosphocreatine ratio are known to activate AMPK (Hardie DG, Salt IP, Hawley SA, Davies SP, "AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge," *Biochem J* 338:717-22, 1999; Hawley SA, Davison M, Woods A, et al., "Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase," *J Biol Chem* 271:27879-87, 1996.) AMPK increases fatty acid oxidation and restricts fatty acid synthesis in an attempt to augment ATP levels in energy-depleted cells. However, in neurons that have a restricted capacity for fatty acid oxidation, this effect could be deleterious (Almeida A, Moncada S, Bolanos JP, "Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway," *Nature Cell Biology* 6: 45-51, 2004).

Inhibition of fatty acid synthase (FAS), the enzyme responsible for the de novo synthesis of palmitate, with C75, a synthetic FAS inhibitor disclosed in U.S. Patent No. 5,981,575 (incorporated herein by reference), increases ATP levels in a number of cell types, including neurons. AMPK is highly expressed in neurons in the hypothalamus, where it appears to play a role in the regulation of food intake. Hypothalamic phosphorylated AMPK (pAMPK) is increased with starvation; C75 treatment inactivates and dephosphorylates AMPK, and induces profound anorexia.

The consequences of AMPK activation in neurons that do not have access to energy supplies is unknown. At present, it is unclear whether AMPK activation during

stress is protective or damaging. There have been no prior studies examining the role of AMPK in stroke.

Summary of the Invention

Applicants have invented a method of neuroprotection which comprises administering a compound to a patient who is experiencing or has experienced a stroke, the compound being an AMPK inhibitor.

Treatments with these agents significantly reduce the size of infarcts, and therefore minimize the loss of brain tissue and neurons. Thus, function can be preserved after stroke or ischemic injury in the brain. Similarly, neuronal loss can be minimized in degenerative diseases that cause neuronal compromise by perturbing energy utilization and availability in neurons.

Detailed Description of the Invention

By "neuroprotection," we mean protecting brain cells, preferably neurons, from permanent damage caused by a stroke during and after the stroke.

By "stroke," we mean an abnormality in brain function resulting from disruption of cerebral circulation.

By "AMPK inhibitor," we mean a compound which inhibits AMPK as determined by the method described by Witters, et al., *Journal of Biological Chemistry*, **267**, pp. 2864-2867 (1992).

Preferably, the AMPK inhibitor is selected from a compound which is not a peptide or other biological (or biologically-derived) material.

The compositions of the present invention can be presented for administration to humans and other animals in unit dosage forms, such as tablets, capsules, pills, powders, granules, sterile parenteral solutions or suspensions, oral solutions or suspensions, oil in water and water in oil emulsions containing suitable quantities of the compound, suppositories and in fluid suspensions or solutions. As used in this specification, the terms "pharmaceutical diluent" and "pharmaceutical carrier," have the same meaning. For oral administration, either solid or fluid unit dosage forms can be prepared. For preparing solid compositions such as tablets, the compound can be mixed with conventional ingredients such as talc, magnesium stearate, dicalcium phosphate, magnesium aluminum silicate, calcium sulfate, starch, lactose, acacia, methylcellulose and functionally similar materials as pharmaceutical diluents or carriers. Capsules are prepared by mixing the compound with an inert pharmaceutical diluent and filling the mixture into a hard gelatin capsule of appropriate size. Soft gelatin capsules are prepared by machine encapsulation of a slurry of the compound with an acceptable vegetable oil, light liquid petrolatum or other inert oil.

Fluid unit dosage forms or oral administration such as syrups, elixirs, and suspensions can be prepared. The forms can be dissolved in an aqueous vehicle together with sugar, aromatic flavoring agents and preservatives to form a syrup. Suspensions can be prepared with an aqueous vehicle with the aid of a suspending agent such as acacia, tragacanth, methylcellulose and the like.

For parenteral administration fluid unit dosage forms can be prepared utilizing the compound and a sterile vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilized before filling into a suitable vial or

ampoule and sealing. Adjuvants such as a local anesthetic, preservative and buffering agents can be dissolved in the vehicle. The composition can be frozen after filling into a vial and the water removed under vacuum. The lyophilized powder can then be scaled in the vial and reconstituted prior to use.

Prior to this invention, the role of neuronal metabolism in the response to brain injury was unknown. With this invention, it is now known that pAMPK is elevated after ischemic stroke in a focal reperfusion model in mice. AMPK is elevated within 90 min of ischemic insult, and increases four-fold during early reperfusion. With this invention it is also now known that, administration of AMPK inhibitors not only resulted in decreased activation of AMPK (as shown by decreased phosphorylation of AMPK), but also provided significant neuroprotection. It has also been found that administration of AMPK activators can exacerbate stroke damage.

While not wishing to be bound by theory, it is thought that AMPK, in addition to playing a major role in physiological energy regulation, has far-reaching effects on cellular survival after ischemic stress. During ischemic stress energy demand is high, yet energy supply is low due to the lack of substrate (oxygen and glucose). By interfering with cellular energy perception and AMPK activation, the ischemic threshold of the cells may be augmented, allowing cells to survive in an energy deficient but quiescent state until energy production can be restored. Modulation of metabolic pathways with synthetic FAS inhibitors/CPT-1 stimulators, such as C75, can alter neuronal metabolism, and create a positive energy balance. C75 inhibits AMPK in both cortical cultures and the hypothalamus in vivo, indicating that AMPK is responsive to neuronal energy balance. C75 administration leads to sustained increases in neuronal

ATP levels in cultured neurons. Under ischemic conditions continued ATP depletion may exceed energy restorative pathways, leading to an inability of the cell to die by the energy-consuming process of apoptosis, shunting cells into necrotic cell death. Thus, sensing ATP levels may be important in neuronal responses to ischemia and AMPK inhibitors may ATP and the neurons.

The invention is further described by the following non-limitative examples:

Experimental Animals. The present study was conducted in accordance with NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the Johns Hopkins University.

Ischemic Model. Cerebral ischemia was induced by 120 minutes of reversible middle cerebral artery occlusion (MCAO) under halothane anesthesia, as previously described by McCullough, et al., *J. Neuroscience* **24**, pp. 257-268 (2004). Rectal muscle temperatures were monitored with a MONO-THERM system and maintained at approximately 37°C during surgery and ischemia. A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a 6.0 nylon monofilament into the internal carotid artery 6mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Animals were awakened from anesthesia and intra-ischemic neurological deficit was confirmed and scored as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no

spontaneous locomotor activity or barrel rolling. If no deficit was observed, the animal was removed from further study. The mice were reanaesthetized after 120 minutes for withdrawal of the suture and subsequent reperfusion. At 22 hours of reperfusion, the brain was harvested for pathological examination. In separate animal cohorts, femoral arterial blood pressure and cortical perfusion (laser Doppler flowmetry) were evaluated throughout MCAO and early reperfusion as previously described McCullough, et al., *J. Neuroscience* **24**, pp. 257-268 (2004). Femoral arterial blood gases, glucose, and hemoglobin were measured at baseline and during occlusion.

Terminal Histopathology: Infarction volume was analyzed by 2,3,5-triphenyltetrazolium staining in five 2-mm slices. Infarction volume was determined by video microscopy and image analysis (Inquiry 3, Loats Associates), as previously described by McCullough, et al., *J. Neuroscience* **23**, pp. 8701-8705 (2003).

Western blot analysis. Tissues were dissected immediately and frozen in liquid nitrogen. Each tissue was homogenized in 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM NaF, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5 mM PMSF, 0.1 mM benzamidine, 50 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor). SDS detergent was added to a final 0.2%, and lysates were boiled for 5 min. After the supernatant was harvested, protein concentration was determined by BCA kit (Bio Rad). For hypothalamus, 40 ug of protein was loaded on the gel. Phosphorylation of AMPKα was determined on a 4-15% gradient SDS-polyacrylamide gel using anti-phospho-AMPKα (Thr172) antibody (1:1000, Cell Signaling) solution (5% BSA, 0,1% TritonX100 in TBS solution) for overnight at 4 °C. Anti-AMPKα antibody (1:1000, Cell Signaling) was used as a loading control (in the

same TBST buffer). Secondary antibody (goat anti-rabbit igG: chemicon) was diluted to 1:5,000 and ECL(pico) detection kit was used for signal.

Immunohistochemistry. Floating brain sections were prepared as previously described (15) with modifications. During perfusion and soaking in sucrose solution, 1 mM NaF was added from 1 M stock solution. Free-floating sections were blocked in PBS containing 5% goat serum, 1mg/ ml BSA, 0.05% Triton-X100, 1 mM NaF for 1hr at room temperature and incubated with anti-phospho-AMPKα antibody (1:100), GFAP (1:1000, DAKO), or NeuN (Chemicon 1:100) in PBS containing 1% goat serum, 1 mg/ml BSA, 0.05% Triton-X100, 1 mM NaF overnight at 4 °C. Secondary antibody (1:200) in 1% goat serum in PBS was incubated for 1 hr 30 min (Texas Red,FITC. Signal was visualized with immunofluorescence confocal microscopy.

Statistical Analysis: All data are expressed as mean ± SEM. Physiological variables and histology were analyzed by 1-way ANOVA with a post-hoc Newman-Keuls to correct for multiple comparisons. Post-ischemic neurological scores were analyzed by the Mann-Whitney U test.

Example 1 - *AMPK is expressed in neurons*

Brain tissue sections obtained from mice subjected to 2 hours of MCAO followed by 2 hours of reperfusion showed prominent neuronal staining for both AMPK and pAMPK (Fig. 1, n=5/gp). They were also stained with NeuN (for mature neurons), GFAP (for astrocytes). Confocal microscopy demonstrates co-localization of NeuN and AMPK/pAMPK, with absent co-lacalization in astrocytes.

AMPK and pAMPK were present in similar levels in nonischemic brain and at 22 hours of reperfusion (data not shown). Confocal microscopy confirmed that AMPK and pAMPK signal localized to neurons, as shown by double–label immunofluorescence using the mature neuronal marker NeuN, but did not co-localize with GFAP. Previous investigations showed strong AMPK signal in activated astrocytes, such as occurs with glial scarring after brain injury (Cox, et al., *Biochem Soc Trans*, **25**, pp. 5583 (1997)). Similar to 4-hour samples, no co-localization of AMPK and GFAP was seen 24 hours after stroke (data not shown). No signal was seen in control sections without the primary antibody.

Example 2 - AMPK is increased after MCAO

Male wild-type (WT) C57Bl6 mice were subjected to right MCAO or sham surgery with varying reperfusion times. Physiological parameters, such as temperature, were held constant. There were no significant differences in blood gas measurements between stroke and sham animals. As can be seen in Fig. 2, pAMPK (as detected by a panα subunit antibody to Thr172 phosphorylation site), was elevated in ischemic brain as early as 90 minutes of ischemia (n=6 per ischemic timepoint; 4 per sham timepoint). pAMPK remained elevated for 12 hours after stroke, then decreased, but was still elevated at 24 hours (22 hours of reperfusion) compared to sham animals. Both hemispheres demonstrated four-fold increases in pAMPK, yet higher levels were found in the contralateral, non-ischemic (NI) left hemisphere. This global effect on pAMPK suggests that metabolic derangements in stroke and compensatory responses are not limited to the ischemic area.

Example 3 - AMPK elevation is seen in the ischemic penumbra.

Assuming that metabolic derangements in stroke may be more severe in the necrotic core of an infarct, whereas anti-apoptotic pathways may be activated in the penumbra in an attempt to salvage marginally functioning brain, we dissected out core and penumbral tissue for Western analysis (see Fig. 3A for areas of ischemia and dissection as illustrated by TTC staining at 24 hr) 6 hr after stroke in rat brain (n=6/gp). As can be seen in Fig. 3B, there were no changes in total AMPK levels, yet pAMPK was dramatically elevated in penumbral tissue and in the contralateral non-ischemic hemisphere. Interestingly pAMPK levels in the core were much lower than in the other areas of brain, only slightly higher than found in sham animals (n=4).

Example 4 - Compound C reduces pAMPK levels and is neuroprotective

To examine the functional effects of AMPK activation in this ischemic model, we explored the effect of stroke outcome in mice treated with compound C, a pharmacological AMPK inhibitor described in *J. Clin Invest* 108, pp. 1167-1174 (2001). Fig. 4A shows the effect on stroke outcome in animals treated with compound C (20mg/kg/body weight delivered intraperitioneally (i.p.); n=14) or vehicle (n=8) at stroke onset (2-hr MCAO with 22 hours reperfusion). Administration of compound C significantly reduced total infarct (as measured by % non-ischemic hemisphere, corrected for edema) as well as striatal (64±5.7 vs. 21±3.9; $p>.001$) and cortical (63±3.4 vs. 19.4±3.3; $p<.001$) infarction volume. A separate non-survival cohort of male mice was utilized for physiological response to compound C. There were no differences in relative cerebral blood flow (CBF) as measured by laser Doppler either intraischemically

(vehicle=9.8±0.54 vs. compound C=10.6±0.9; % of baseline; p=n.s.), or during 30 minutes of reperfusion. There were no significant differences in physiological parameters such as mean arterial pressure (MAP), blood glucose, hemoglobin (Hg), pH, PO_2, or PCO_2 (Table 1).

Additional groups of vehicle or compound C treated mice subjected to either 2-hour MCAO (n=6/gp) or sham surgery (n=4/gp) were sacrificed at 4 hr (Fig. 4B) or 24 hr (Fig. 4C) after stroke for determination of AMPK and pAMPK levels. The anticipated increase in pAMPK at 2 hr of reperfusion was seen in vehicle treated animals, and reduced at 2 hr in compound C treated animals in both sham and stroke animals, demonstrating that compound C reduced pAMPK levels, while total AMPK levels were unchanged. The effect of compound C was transient, with pAMPK levels returning to those seen in vehicle and compound C-treated mice by 24 hr.

Example 5 - Reduction in pAMPK levels by the FAS inhibitor C75 is neuroprotective in stroke.

We administered the FAS inhibitor C75 (20 mg/kg i.p.) or vehicle (RPMI) immediately prior to the onset of ischemia (n=9/gp). We based this dose on previous in vivo data that demonstrated an effect on feeding behavior and an inhibition of C75 activity in hypothalamus. As shown in Fig.5A, there was a significant reduction in total and regional infarction volume seen in the C75-treated group (P<.001). There were no significant differences in intra-ischemic or reperfusion blood flow blood flow as measured by laser Doppler nor were there any differences seen in MAP, pH or blood gas measurements in the non-survival cohort (n=4, data not shown). Additional groups of vehicle or C75 treated mice subjected to either 2-hour MCAO (n=6/gp) or sham

surgery (n=4/gp) were sacrificed at 4 hr (Fig 5B) or 24 hr (Fig. 5C) after stroke for analysis of AMPK and pAMPK levels. The anticipated increase in pAMPK at 2 hr of reperfusion was seen in vehicle and C75-treated mice. Reductions in pAMPK levels were seen in C75 treated animals at 24 hrs in both sham and stroke mice, documenting the more lasting central effect of C75 (compared to compound C) in reducing pAMPK.

Example 6 - AMPK activation with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) exacerbates stroke.

The AMPK activator AICAR was administered at stroke onset (500mg/kg; n=11/gp). There was a small but significant ($p>.05$) exacerbation of total and cortical stroke volumes in AICAR-treated animals. No differences were seen in the striatum. Physiological monitoring demonstrated a significant drop in MAP in AICAR treated animals that self-corrected by 15 minutes after injection (MAP Vehicle; 84 vs. AICAR; 69 mmHg, $P>.05$). This reduction in MAP could have contributed to the increase in stroke damage in AICAR-treated mice due to decreased vascular tone and underperfusion of peri-infarct areas. There were no differences in intraischemic CBF, but a reduction in CBF was seen in AICAR-treated mice at 30 minutes after reperfusion (98 vs. 82.7; % of baseline, Fig 6D). Western analysis showed small elevations of pAMPK protein at 2 hours that returned to baseline by 24 hours.

Table 1: Intra-ischemic physiological variables and Laser Doppler Flow (LDF) values in mice in each of the experimental groups (n=4/group). There were no significant differences in physiological measurements between each drug group compared to respective vehicle in C75 and Compound C treated animals. MAP and LDF are shown averaged over the 2 hours of ischemia. AICAR treated animals had a significant reduction in MAP at 15 minutes ($p<.05$), with no overall difference over 2 hours of monitoring. Reductions in intraischemic LDF were equivalent in Vehicle and AICAR animals, however there was a significant reduction ($p<.05$) in LDF at 30 minutes of reperfusion.

	Vehicle	**Compound C**	**Vehicle**	**C75**	**Vehicle**	**AICAR**
Ph	7.37±0.05	7.36±0.07	7.30±0.10	7.34±0.08	7.37±0.05	7.33±0.11
PCO_2	45.2±2.8	42.1±3.1	47.2±4.7	47±7.1	42±3.6	39.7±4.9
PO_2	140±9.8	144.6±14.3	144.7±11.6	135±14.2	147±15.9	128±15.2
MAP	80.7±4.8	82.2±5.9	81.5±3.9	79.1±6.5	84±7.1 @15 81.6±4.4 (all)	69±12.4@15 77.7±7.3 (all)
Hg	13.6±1.9	12.7±1.3	13.8±1.8	12.9±0.5	13.8±2.3	13.4±0.9
HCO_3	23.5±1.1	23.1±.08	22.6±2.4	22.8±1.2	23.6±2.6	22.9±2.3
LDF (%BL)	9.8±1	10.6±0.9	10±1.3	10.6±0.88	11.3±.8 RP 98±5.3	11.1±1.2 RP 83±10.8

We claim:

1. A method of neuroprotection which comprises administering a compound to a patient who is experiencing or has experienced a stroke, the compound being an AMPK inhibitor.

2. The method of claim 1, wherein the compound is not a peptide or other biological or biologically-derived material.

3. The method of claim 2, wherein the compound is C75.

4. The method of claim 2, wherein the compound is Compound C.

5. The method of claim 2, wherein the compound is not C75 or Compound C.

6. A pharmaceutical composition for the treatment of stroke, comprising an AMPK inhibitor.

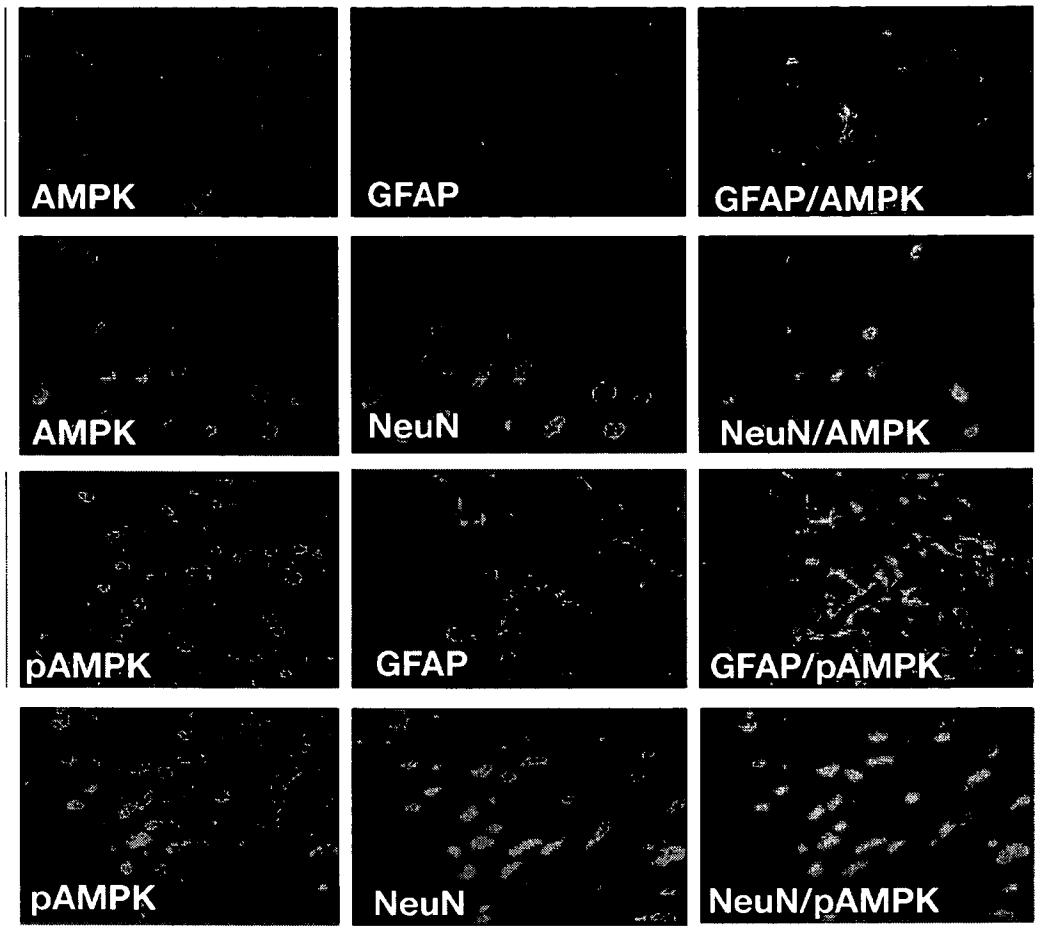


Fig. 1

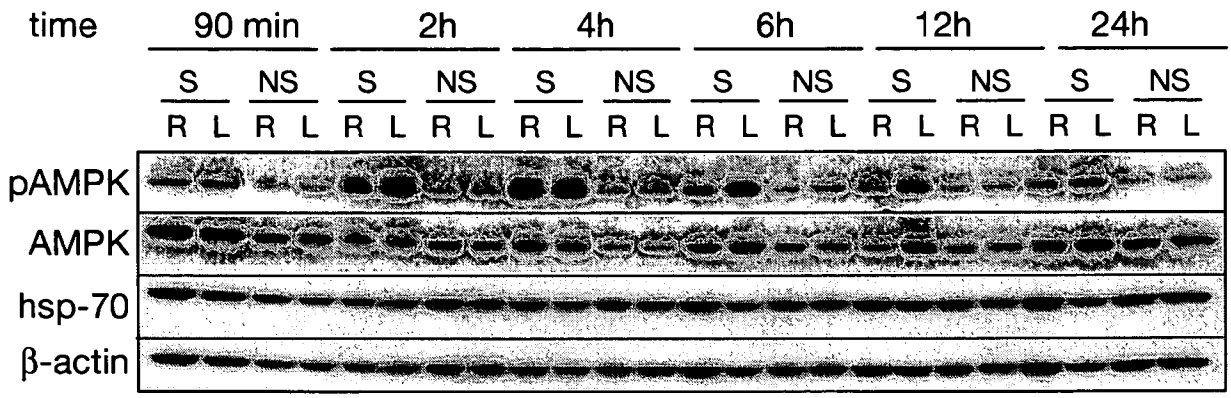
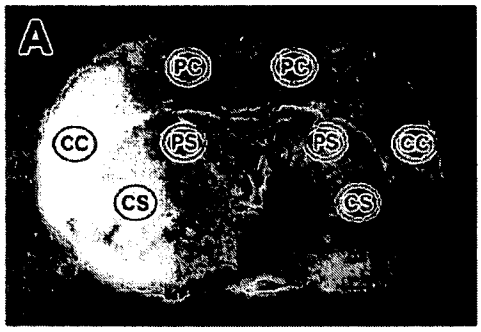


Fig. 02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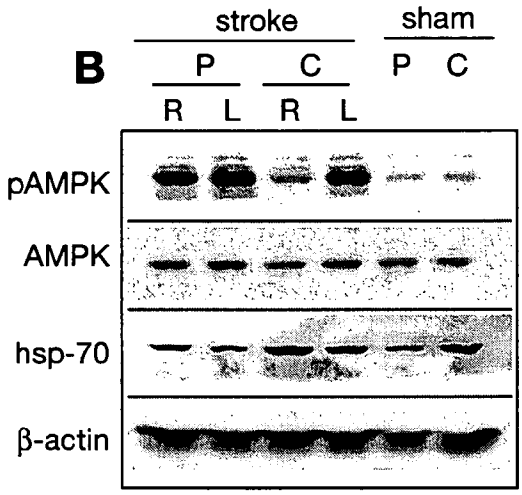
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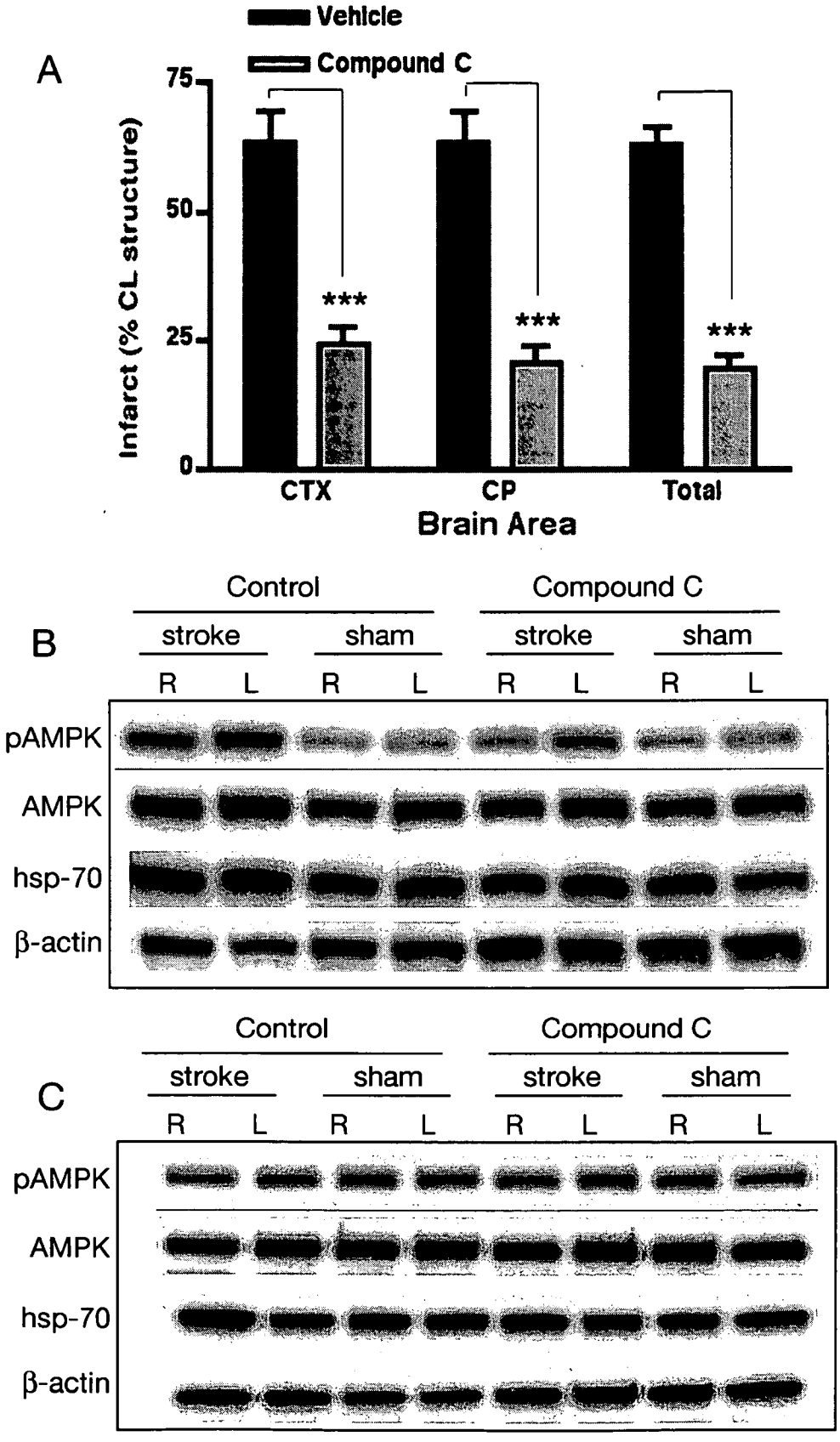
Fig. 03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β-actin

Fig. 04

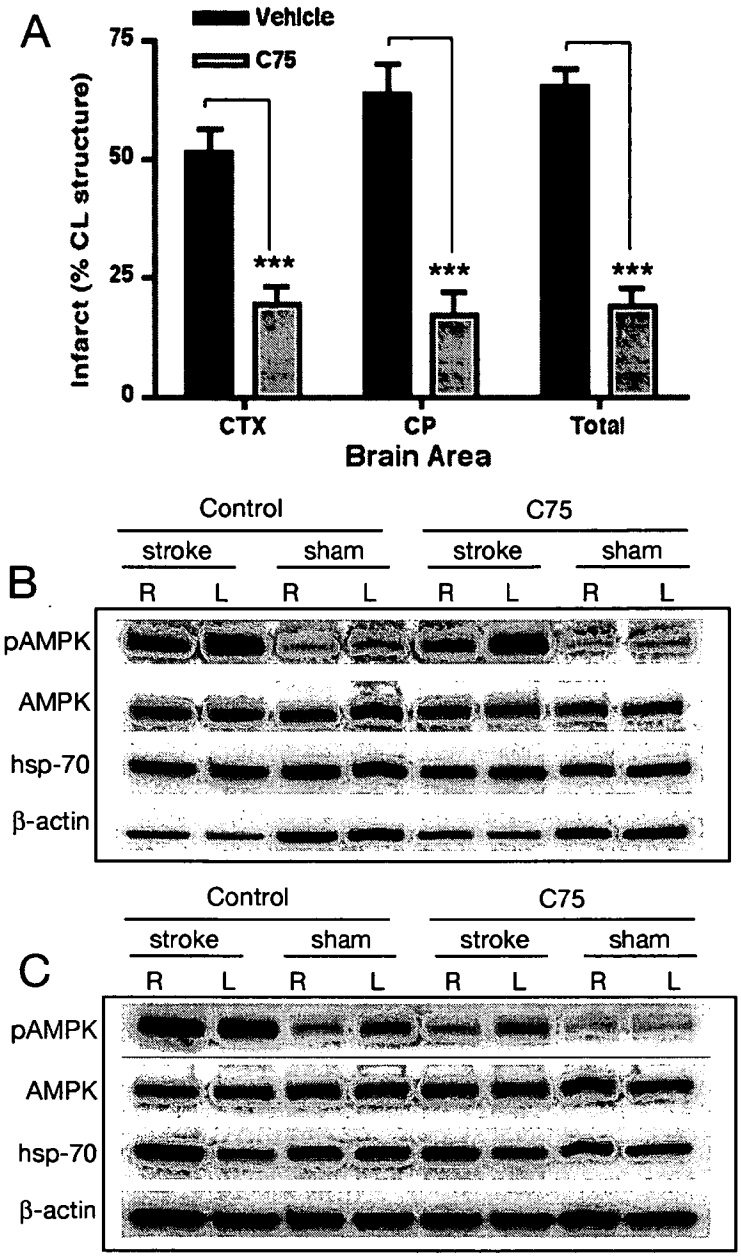
A
Vehicle
C75
75
50
25
0
Infarct (% CL structure)
***
***
***
CTX
CP
Total
Brain Area
B
Control
C75
stroke
sham
stroke
sham
R
L
R
L
R
L
R
L
pAMPK
AMPK
hsp-70
β-actin
C
Control
C75
stroke
sham
stroke
sham
R
L
R
L
R
L
R
L
pAMPK
AMPK
hsp-70
β-actin

Fig. 05

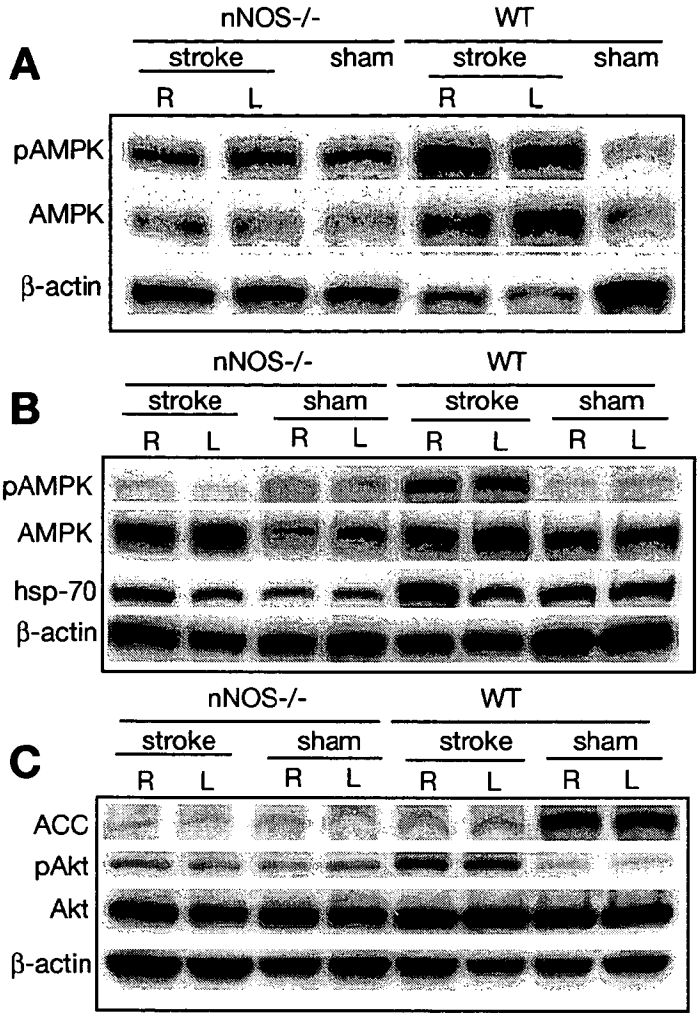
A
nNOS-/-
WT
stroke
sham
stroke
sham
R
L
R
L
pAMPK
AMPK
β-actin
B
nNOS-/-
WT
stroke
sham
stroke
sham
R
L
R
L
R
L
R
L
pAMPK
AMPK
hsp-70
β-actin
C
nNOS-/-
WT
stroke
sham
stroke
sham
R
L
R
L
R
L
R
L
ACC
pAkt
Akt
β-actin

Fig. 07